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<b>(54) Title:</b> NEVRON GROWTH INHIBITORY MOLECULES OR DERIVATIVES THEREOF USED TO IMMUNIZE MAMMALS THEREBY PROMOTING AXON REGENERATION		
<b>(57) Abstract</b> <p>This invention pertains to compositions comprising immunogenic agents having neuron growth inhibitory properties, which cause polyreactive antibodies to be generated that bind to axon growth inhibitors, thereby promoting axon regeneration. Said agents include myelin-associated glycoprotein (MAG), arretin or proteoglycan proteins. These compositions can be used to restore nerve transmission, particularly motor and sensory function, after spinal cord lesions or optic nerve damage. They can also be used to treat diseases involving axonal damage, such as Multiple Sclerosis and stroke. These compositions can be used further as prophylactic vaccines prior to onset of CNS disease or injury.</p>		

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# **NEURON GROWTH INHIBITORY MOLECULES OR DERIVATIVES THEREOF USED TO IMMUNIZE MAMMALS THEREBY PROMOTING AXON REGENERATION**

## **FIELD OF THE INVENTION**

This invention pertains to the field of nerve regeneration.

## **BACKGROUND OF THE INVENTION**

Following trauma and disease in the adult mammalian CNS, injured neurons do not regenerate their damaged axons. Axon growth inhibitory activity is an important cause of the failure of axon regeneration in the central nervous system (CNS). Axon growth inhibitors are predominantly associated with CNS myelin and constitute an important barrier to regeneration. In particular, axon growth inhibitory activity is present in CNS myelin and the plasma membrane of oligodendrocytes, which synthesize myelin in the CNS (see Schwab *et al.*, (1993) *Ann. Rev. Neurosci.* 16:565-595 for review).

Axons and dendrites grow out via growth cones located at their tips. The growth follows specific pathways that are delineated by cells and extracellular matrix that are located along the way. The guidance of the growth depends on various classes of adhesion molecules, on intercellular signals, as well as on factors that repel and inhibit growth cones. The growth cone located at the end of a rapidly growing neurite grows about 1 mm per day. The cone consists of a broad and flat expansion, with numerous long microspikes or filopodia that extend like fingers. These filopodia are continually active. Some filopodia retract back into the growth cone. Other filopodia continue to elongate and wave around, touch and adhere to the substratum. The webs or veils between the filopodia form lamellipodia.

The growth cone can explore the area that is ahead of it and on either side with its lamellipodia and filopodia. When such an elongation comes in contact with a surface that is unfavorable, it withdraws. When such an elongation comes in contacts with a surface that is favorable, it persists

longer and can steer the growth cone moving it in that direction. Hence, the growth cone can be guided by subtle variation in surface properties of the substrata.

Two important classes of positive growth-signaling molecules are the immunoglobulin superfamily (eg. N-CAM) and the Ca<sup>2+</sup>-dependent cadherin family(eg. N-cadherin). Both families are usually present on the surface of growth cones, axons, and other cell types that interact with such cones. These include glial cells found in the CNS as well as muscle cells in the periphery of the body. Growth cones can also move over various components of the extracellular matrix (eg. laminin), which they bind to via cell-surface matrix receptors found in the integrin family.

The importance of a given cell-cell or cell-matrix adhesion molecule can be shown by blocking its function with an antibody, and observing any disturbances that occur to axon outgrowth. However, a growth cone can utilize several adhesion systems to migrate, and antibodies against any single one of them may have little effect. It is only when multiple antibodies are applied (ie. to block all of them together) that the growth cone's growth is hindered. Various combinations of adhesion molecules allow for greater variety in the surface properties of growth cones, thereby allowing for subtle and complex pathway selection according to the combinations of molecules on the surfaces of cells.

The collapse of an axon growth cone is an important response of the growing axon to inhibitory cues in the environment. Collapse of the lamellipodium is sometimes followed by retraction of the neurite (Kapfhammer and Raper (1987) *J. Neurosci.* 7:201-212,; Raper and Grunewald (1990) *Exp. Neurol.* 109:70-74; Bandtlow *et al.* (1990), *J. Neurosci.* 10:3837-3848). Many previously characterized inhibitory molecules found in the developing nervous system have been shown to cause growth cone collapse *in vitro* (Davies *et al.*, (1990) *Neuron* 4:11-20; Stahl *et al.*, (1990) *Neuron* 5:735-743; Bandtlow *et al.*, (1990); Keynes *et al.*, (1991) *Ann. N.Y. Acad. Sci.* 633:562; Luo *et al.*, (1993) *Cell* 75:217-227).

At present, the myelin associated inhibitors appear to be a major contributor to the failure of axon regeneration in the CNS *in vivo*. Other non-myelin associated axon growth inhibitors in the CNS may play a lesser role. Furthermore, inhibitors that are not derived from myelin and are

associated with the scar at the site of a CNS lesion may also play a role in blocking some of the axon regeneration after injuries.

### **Growth Inhibitors in the CNS Associated with Myelin:**

*NI 35/250:*

Schwab and his colleagues have reported the characterization of an axon growth inhibitor found in CNS myelin (Schwab *et al.*, (1993) *Annu. Rev. Neurosci.* 16:565-595). To date the molecular identity of the 35 and 250 kDa inhibitors has not been reported.

#### *Myelin-Associated Glycoprotein (MAG):*

Recently, myelin-associated glycoprotein (MAG) was identified as an inhibitor of axon growth (Mckerracher *et al.*, (1994) *Neuron* 13:229-246 and 805-811; Mukhopadhyay *et al.*, (1994)). MAG is a large multifunctional glycoprotein (100kDa) that has both adhesive (Johnson *et al.*, (1989) *Neuron* 3:337-385) and inhibitory functions. There is some evidence that the adhesive interactions of MAG may be mediated via members of the sialoadhesin family (Kelm *et al.*, (1994)). The identity of the receptor(s) mediating inhibition is not yet known.

Anion (DEAE) exchange chromatography of detergent extracts of bovine CNS myelin yields two major peaks of neurite growth inhibitory activity (Mckerracher *et al.*, (1994) *Neuron* 13:229-246 and 805-811). The first peak was shown to be enriched in MAG. Direct evidence that MAG has neurite growth inhibitory activity was provided by testing recombinant MAG *in vitro* (Mckerracher *et al.*, (1994) *Neuron* 13:229-246 and 805-811; Li *et al.*, (1996) *J. Neurosci. Res.* 46:404-414). It has also been shown that MAG-coated polystyrene beads cause the collapse of growth cones of neonatal rat hippocampal neurons (Li *et al.*, (1996) *J. Neurosci. Res.* 46:404-414; Shibata *et al.*, (1996a)). This MAG-induced growth cone collapse like that which occurs on contact with oligodendrocytes is not associated with increases in intracellular Ca<sup>++</sup> (Shibata *et al.*, (1996a); Kater *et al.*, (1996) *Soc. Neurosci. Abst.* 26:583.10).

The collapse induced by MAG is specific for axonal but not dendritic growth cones (Shibata *et al.*, (1996a)). Furthermore, CHO cells expressing MAG on their surface are an inhibitory

substrate for neurite growth from a variety of primary neurons from the CNS and PNS as compared to mock transfected cells (Mukhopadhyay *et al.*, (1994); DeBellard *et al.*, (1996)).

*Arretin:*

Another growth inhibitory protein has been identified, termed arretin (Xio *et al.*, (1994) *Soc. NeuroScience Abstract.* vol. 23). The apparent molecular weight of arretin is approximately 70 kDa, and it has potent growth inhibitory activity when plated as a substrate. Arretin causes growth cone collapse, and recent work has shown that the 70 kDa component consists of at least two separate inhibitors. Monoclonal antibodies raised against these inhibitors are able to block neurite growth on myelin and extracts of myelin.

*Tenascins:*

Four members of the tenascin family have been identified and characterized: tenascin-C, tenascin-R, tenascin-X and tenascin-Y (Bristow *et al.*, *Cell Biol.*, 122, 265-278, 1993; Erickson, H.P., *J. Cell Biol.*, 120, 1079-1081, 1993). Tenascin-X and tenascin-Y are not prominent in the nervous system. Studies have indicated that both tenascin R and tenascin C are minor inhibitory components of octylglucoside extracts of myelin. These data suggest that growth inhibitory proteins from the CNS matrix may become associated with isolated myelin fragments.

Tenascin-C is important in the development of the nervous system and it is the best characterized member of this protein family. It is generated by alternative splicing (Weller *et al.*, *J. Cell Biol.*, 112, 355-362, 1991; Sriramarao and Bourdon, *Nucl. Acids Res.*, 21, 347-362, 1993) and the variants are expressed both in the nervous system and in several non-neural tissues. Tenascin-C has been suggested to be involved in neuron-glia adhesive and migratory events and to promote axon outgrowth after injury of peripheral nerves.

Tenascin-R (TN-R), has a modular structure similar to TN-C, previously designated J1-160/180 and janusin in rodents, or restriction in chicken (Pesheva *et al.*, *J. Cell Biol.*, 109, 1765-1778, 1989; Fuss *et al.*, *J. Neurosci. Res.*, 29, 299-307, 1991, and *J. Cell Biol.*, 120, 1237-1249, 1993). Tenascin-R is predominantly expressed by oligodendrocytes during the onset and early phases of myelin formation and remains detectable in myelin-forming oligodendrocytes in the adult, and is

also expressed by neurons (Pesheva *et al.*, 1989; Fuss *et al.*, 1993). Tenascin-R has been shown to be involved in promotion of neurite outgrowth and morphological polarization of differentiating neurons when presented as a uniform substrate (Lochter and Schachner, *J. Neurosci.*, 13, 3986-4000, 1993; Lochter *et al.*, *Eur. J. Neurosci.*, 6, 597-606, 1994). When offered as a sharp substrate boundary with a neurite outgrowth conducive molecule, tenascin-R is repellent for growth cone advance (Taylor *et al.*, *J. Neurosci. Res.*, 35, 347-362, 1993; Pesheva *et al.*, 1993).

### **Growth Inhibitors in the CNS Not Primarily Associated with Myelin:**

#### *Chondroitin Sulfate Proteoglycans (CSPGs):*

Proteoglycans (PGs) are proteins that are found predominantly on the cell surface and in the extracellular matrix; they are covalently bound to complex carbohydrates called glycosaminoglycans. Glycosaminoglycans (GAGs) are polymers of disaccharide repeats, which are mostly highly sulphated and negatively charged. The main glycosaminoglycans in PGs are chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate. (Ruoslahti, E., *Ann. Rev. Cell Biol.*, 4, 229-255, 1988). The number of GAG chains can vary from one to over one hundred.

Chondroitin sulfate proteoglycans (CSPGs) constitute the major population of PGs in the CNS. The different patterns of localization and developmental expression of CSPGs throughout the nervous system implicate them in diverse roles in development and in regeneration. After injuries in the adult CNS, CSPGs are thought to be important in the formation of the glial scar. They have been implicated as both positive and negative modulators of axonal growth (Faissner *et al.*, *J. Neurochem.*, 54, 1004-1015, 1994). However, NG2, an integral membrane CSPGs expressed on the surface of glial progenitor cells, inhibits neurite growth. The NG2 proteoglycan also inhibits neurite growth after digestion with chondroitinase ABC, indicating that the inhibitory activity is a property of the core protein and not the covalently attached chondroitin sulfate glycosaminoglycan chains (Dou and Levine, *J. Neurosci.*, 14, 7616-7628, 1994), but for many other types of CSPGs the inhibitory activity resides in the glycosaminoglycan.

This highly sulfated proteoglycan which is a potent inhibitor of neurite growth *in vitro* (Snow *et al.*, *Neurol.*, 109, 111-130, 1990), has been shown to be involved in the differentiation of developing retinal ganglion cells, and by acting as an inhibitory substrate serves to appropriately guide ganglion cell axons toward the optic disc (Brittis and Silver, *Proc. Nat. Acad. Sci. USA.*, 19, 7539-7542, 1992). McKeon *et al.*, (1991) *J. Neurosci.* 11:3398-411 have reported that astrocytes harvested from the site of cerebral cortical lesions express increased amounts of CSPG, which reduces neurite growth on these cells *in vitro*. The expression of CSPG on the surface of a subset of cultured astrocytes has also been shown to correlate with their reduced capacity to support neurite growth (Meiners *et al.*, *J. Neurosci.*, 15, 8096-8108, 1995). Because proteoglycans are a very heterogeneous class of proteins with diverse biological activities it is essential that individual, identified proteins be considered.

#### *Phosphacan:*

Phosphacan is a proteoglycan in brain recognized by the 3F8 antibody (Maurel *et al.*, *Proc. Nat. Acad. Sci. USA*, 91, 2512-2516, 1994), and by the 6B4 antibody (Maeda *et al.*, *Neurosci.*, 67, 23-35, 1995). Phosphacan is a splice variant of a receptor-type protein tyrosine phosphatase, although phosphacan itself lacks the phosphatase domains. It is a protein with an apparent molecular weight of approximately 500 kDa, having a core glycoprotein of approximately 400 kDa. The HNK-1 monoclonal antibody recognizes a 3-sulphated carbohydrate epitope, and this epitope is strongly represented in phosphacan from 7-day brain, but not in adult brain (Rauch *et al.*, *J. Biol. Chem.*, 266, 14785-14801, 1991). In development phosphacan is immunostained on radial glia and on neurons (Maeda *et al.*, 1995) and generally it is expressed in both white matter and grey matter regions (Meyer-Puttlitz, *et al.*, *J. Comp. Neurol.* 366, 44-54, 1996). and therefore, unlike the myelin inhibitors, it is not localized only to white matter areas. It appears to be synthesized only by astroglia (Engel *et al.*, 1996).

#### *Versican:*

Versican, a CSPG originally isolated from fibroblasts, also called PG-M, has an apparent molecular weight of approximately 900 kDa, with a core protein of approximately 300 to 400 kDa (Braunewell *et al.*, *Eur. J. Neurosci.*, 7, 792-804, 1995; Naso *et al.*, 1994). Versican belongs to a family of aggregating CSPGs; other members of the family include the cartilage-derived



aggrecan, and two PGs expressed in the nervous system, neurocan and brevican (Dours-Zimmermann and Zimmermann, *J. Biol. Chem.*, 269, 32992-32998, 1994). Versican is widely distributed in adult human tissues, associated with connective tissue of various organs, in certain muscle tissues, epithelia, and in central and peripheral nervous tissues. Four versican isoforms are known (Vo, V1, V2, V3), derived by alternative splicing. They vary in calculated mass from approximately 370 kDa (Vo) to approximately 72 kDa (V3). It has been suggested that the association of versican expression with cell migration and proliferation *in vivo* and its adhesion inhibitory properties *in vitro* point to pathological processes such as tumorigenesis and metastasis (Bode-Lesniewska *et al.*, *Histol. & Cyto.*, 44, 303-312, 1996; Naso *et al.*, *J. Biol. Chem.*, 269, 32999-33008, 1994).

Other CSPGs related to versican are brevican (Mr approximately 145 kDa) and neurocan (Mr > 300 kDa). Neither of these is known to be expressed by oligodendrocytes and are therefore not expected to be present in CNS myelin (Engel *et al.*, *J. Comp. Neurol.* 366, 34-43, 1996; Yamada *et al.*, *J. Biol. Chem.*, 269, 10119-10126, 1994).

Another CSPG family member that is not related to either versican or phosphacan, is NG2. Although it is expressed by O2A progenitor cells in the developing rat nervous system, it has no apparent homology to arretin-relevant CSPG=s, and has an Mr approximately 400-800 kDa with a core protein of approximately 300 kDa (Nishiyama *et al.*, *J. Cell Biol.*, 114, 359-371, 1991).

#### **Prior Strategies for Overcoming Growth Inhibition:**

It is well documented that adult neurons cannot extend neurites over CNS myelin *in vitro* or over proteoglycans derived from the glial scar. While embryonic astrocytes support axonal growth, astrocytes isolated from the glial scar inhibit axon growth. This is because they express inhibitory proteoglycans (McKeon *et al.*, (1991) *J. Neurosci.* 11:3398-411). Also, it is well established that various types of proteoglycans are present in the adult nervous system (see above), and thus the proteoglycan matrix of the adult CNS is an important barrier to axonal regrowth after injury.

The growth inhibitory properties of CNS myelin have been demonstrated in a number of different laboratories by a wide variety of techniques, including plating neurons on myelin substrates or cryostat sections of white matter, and observations of axon contact with mature oligodendrocytes (Schwab *et al.*, (1993) *Annu. Rev. Neurosci.* 16:565-595 ).

It has also been well documented that removing myelin *in vivo* improves the success of regenerative growth over the native terrain of the CNS. For example, regeneration has been demonstrated to occur after irradiation of newborn rats, a procedure that kills oligodendrocytes and prevents the appearance of myelin proteins (Savio and Schwab (1990) *Neurobiology* 87: 4130-4133). After such a procedure in rats and combined with a corticospinal tract lesion, a few of the corticospinal axons regrow long distances beyond the lesions.

It is now clear that multiple inhibitors that block axon growth are present in myelin. Most strategies to block inhibition have focussed on blocking individual inhibitors with monoclonal antibodies.

For example, Schwab *et al.*, generated a monoclonal antibody to an inhibitor which recognizes two proteins on Western blots with molecular weights of 35 and 250 kDa. *In vitro* assays have demonstrated that this antibody was able to block some of the neurite growth inhibitory activity of CNS myelin. This monoclonal antibody was introduced by either being infused directly via osmotic pumps or by transplanting the hybridoma cells into young adult rats was shown to stimulate the growth of a small number of corticospinal tract axons after lesions of the spinal cord (Schnell & Schwab (1990) *Nature* 343:269-272). In other experiments raphe neurons projecting to the spinal cord were also shown to regenerate after spinal lesions which lead to improvement of some locomotor function (Bregman *et al.*, (1995) *Nature* 378: 498-501). A striking aspect of these *in vivo* studies on NI 35/250 is that only a very small number of axons regenerate after treatment with this monoclonal antibody. This suggests that other axon growth inhibitors exist and need to be blocked before effective regeneration of large numbers of axons can be achieved.

In addition, a chick model of spinal cord repair has been used to demonstrate the onset of myelination correlates with the loss of the regenerative ability of the axons (Keirstead *et al.*, (1992) *Proc. Nat. Acad. Sci. (USA)* 89:11664-11668). The removal of myelin with anti-galactocerebroside and complement in the embryonic chick spinal cord extends the permissive period for axonal regeneration. These experiments demonstrate a good correlation between myelination and the failure of axons to regenerate in the CNS. This approach, however, has thus far not been successful in removing myelin in adult rodent CNS.

Other strategies to foster axonal regrowth in injured spinal cord include attempts to bypass myelinated CNS tracts with non-CNS tissue such as peripheral nerve grafts (David & Aguayo (1981) *Science* 214:931-933; Cheng *et al.*, (1996) *Science* 273:510-513). Another strategy is to remove myelin (Keirstead *et al.*, (1992) *Proc. Natl. Acad. Sci.* 89:11664-11668). A further strategy is to use monoclonal antibodies to block specific myelin-associated inhibitors of axon growth (Schnell & Schwab (1990) *Nature* 343:269-272; Bregman *et al.*, (1995) *Nature* 378: 498-501).

In a model of demyelinating disease, it has been demonstrated that myelin repair can be stimulated by the passive administration of a polyreactive monoclonal antibody (Hunter *et al.*, (1997) *J. Neurol. Sci.* 150:103-113; Miller *et al.*, (1994)). A monoclonal polyreactive antibody called SCH94.03 has been isolated following immunization of mice with spinal cord homogenate. This antibody augments repair of myelin after intracerebral inoculation of genetically susceptible mice with TMEV, a pathogenic virus that produces immune-related CNS demyelination (Miller *et al.*, (1994)). These investigators have also shown that this antibody can pass through the blood-brain barrier and enter the CNS tissue. Furthermore, polyreactive antibodies generated following immunization with spinal cord homogenate tend to be polyreactive against proteins expressed in myelin (see Asakura *et al.*, (1995)), likely due to the highly charged nature of many of these molecules.

Each of these targeted-antibody approaches, however, requires the identification of all the major inhibitors and generating function blocking antibodies against each of them. For effective axon regeneration to occur, it will be important to block all of the growth inhibitors in CNS tissue.

Although the myelin-associated inhibitors are thought to contribute importantly to the failure of axon regeneration, other inhibitors associated with other components of the CNS may also play a role. Some of these inhibitors may also be associated with the astrocytes in the white matter (myelinated) tracts of the CNS. These may also contribute to the failure of axon regeneration.

Thus, a need remains in the art for a procedure of overcoming a sufficient portion of the inhibitory signals, to allow for growth of axons in the CNS.

Accordingly, it is an object of this invention to provide immunogenic compositions that stimulate a polyreactive antibody response in a subject in order to block axon growth inhibitors that prevent regeneration.

## SUMMARY OF THE INVENTION

The present invention relates to regeneration of central nervous system (CNS) axons in mammals. Specifically, the present invention relates to compositions comprising immunogenic agents derived from neuron growth inhibitory molecules and the method of administering this composition a mammal to cause polyreactive antibodies to be generated *in vivo*, that bind to axon growth inhibitors, thereby promoting axon regeneration.

In one embodiment of the present invention, the immunogenic agents are derived from mammalian central nervous system tissue homogenate. The central nervous system tissue may comprises one or more components of the group comprising spinal cord, brain, brainstem, and optic nerves.

In another embodiment, the immunogenic agents are derived from mammalian CNS tissue that is largely myelin.

In a further embodiment of the present invention, the immunogenic agents are derived from mammalian peripheral nervous system tissue homogenate.

In yet a further embodiment of the present invention, the immunogenic agents are purified or recombinant proteins, or fragments or derivatives thereof. Examples of specific immunogenic agents that may be used include MAG, arretin, and proteoglycans. These proteins may be used alone or in combination.

In yet another embodiment, the immunogenic agents are provided by gene therapy.

The immunogenic agents may be derived from any mammal, but are preferably human.

Another embodiment of the present invention provides for a kit comprising the immunogenic compositions and instructions for use in immunization and neuron regrowth.

In yet another embodiment, the present invention describes methods of promoting axon repair and/or regeneration in a subject by blocking axon growth inhibitors, comprising immunizing said subject with therapeutically effective amounts of an immunogenic composition. This immunization may be carried out in conjunction with other known therapies, including cell or tissue transplants, surgical resections to remove scar tissue, and the administration of neurotrophin or growth factors to stimulate the growth state of neurons. These methods may be used to promote repair following injuries, whether acute or chronic, such as spinal cord injuries, optic nerve injuries, and injuries to other white matter tracts. These methods may also be used when a subject requires repair and/or regeneration due to disease, such as strokes, multiple sclerosis, diseases of the optic nerve, and other conditions in which damage to CNS axons occur.

In a further embodiment, the present invention describes the use of immunogenic compositions as prophylactic vaccines.

Various other objects and advantages of the present invention will become apparent from the detailed description of the invention.

## **BRIEF DESCRIPTION OF THE FIGURES**

**Figure 1.** Regeneration of corticospinal tract fibers in the injured adult mouse spinal cord. (a.) Micrograph of the lesioned corticospinal tract in a mouse immunized with spinal cord homogenate in IFA. WGA-HRP labels the tract rostral (toward the left) and caudal (toward the right) to the lesion (arrow). Many regenerated axons can be seen caudally. (b.) Higher magnification of an adjacent section showing the scar at the site of lesion. WGA-HRP-labeled axons can be seen coursing through the scar (arrow; same area shown at higher magnification in insert). Large number of HRP-labeled fibers that have regenerated caudal to the lesion (arrowhead) have bypassed the scar. (c.) Dark-field micrograph showing a bundle of regenerated axons (between arrows) 7.5 mm caudal to the lesion. (d.) Montage of two adjacent tissue sections from another animal in which most of the WGA-HRP labeled fibers in the tract course through the area of lesion (asterisk). Caudal is located to the right side of the lesion. (e.) A bright-field image of one of the sections from which the montage in Ad@ was made. Note the lesion at the centre (asterisk) which is seen as the area of high cellularity. (f.) Neurons retrogradely labeled with Fluorogold in the motor cortex in a mouse immunized with bovine myelin in IFA. (g.) The same field as in Af@ showing neurons double-labeled with Fluororuby. Scale bar in a=500  $\mu$ m; b = 200  $\mu$ m; c=100  $\mu$ m; d, e = 200  $\mu$ m; f, g & insert = 50  $\mu$ m.

**Figure 2.** Percentage of double-labeled neurons retrogradely labeled with Fluorogold and Fluororuby. This is an index of the proportion of corticospinal tract neurons that regenerated their axons.

**Figure 3.** Recovery of motor function in immunized mice. Recovery of motor control of the foot and toes assessed three weeks after lesioning in a group of mice immunized with spinal cord homogenate or controls (injected with IFA only) using the following tests: (1) spreading and closing of the toes, and (2) stepping of the hind-limb in response to stroking of the dorsum of the foot. About 66% (n=12) of the treated animals were able to spread and close the toes of the hind limbs as compared to only 10% (n=10) of control mice. Stepping with the hind-limbs after stroking of the dorsum of the foot was detected in 58% of the immunized mice but in none of the control mice. These results indicate that the anatomical regeneration leads to functional recovery. The slightly higher percentage of the latter may be due to sprouting from unlesioned descending tracts as previously shown.

**Figure 4.** Levels of serum immunoglobulin in immunized and control mice. Enzyme-linked immunosorbent assays shows that the levels of IgG (a) and IgM (b) in sera of mice immunized with mouse spinal cord homogenate (IM) is higher than in controls (C). (c.) Sera from immunized mice also show strong myelin reactivity as compared to sera from controls.

**Figure 5.** Assessment of myelin-reactive antibodies in immunized mice. (a.) Western blot of spinal cord proteins showing an example of the binding of the sera from an immunized mouse (left lane) and a control mouse (right lane) both of which were sacrificed 3 weeks after spinal cord hemisection. The antibodies in the sera of immunized mice bound variably to multiple bands (not yet identified) as compared to controls. MW markers (arrowheads): 103, 76, and 49 kDa. (b.) Staining for immunoglobulin in tissue sections of the spinal cord from a mouse immunized with mouse spinal cord homogenate in IFA and sacrificed 3 days after hemisection. Micrograph taken through the dorsal column shows strong labeling of myelin. (c.) A similar area through the spinal cord of a control mouse taken 3 days after hemisection. (d.) Toluidine blue stained Epon embedded section of the cervical spinal cord of an immunized mouse shows no evidence of demyelination or infiltration of immune cells into the CNS or sub-meningeal space. Axonal degeneration is only seen in the fasciculus gracilis whose fibers were severed by the lower thoracic cord hemisection. (e.) Higher magnification of ventral white matter shows no evidence of inflammatory changes. Scale bar in b, c = 50  $\mu$ m; d = 200  $\mu$ m; e = 100  $\mu$ m.

**Figure 6.** The technique used to study axon regeneration in the optic nerve of rats after immunization. (a) The retinal ganglion cell axons in the optic nerve are transected by constriction with 10.0 sutures. (b) Other treatments, such as the application of neurotrophins, C3 enzyme or other agents that help stimulate regeneration can be added to the site of injury to act synergistically with the immunization approach. (c) Regenerating axons are detected by anterograde labeling after injection of tracer into the eye.

**Figure 7.** Immunization controls. The top panel shows longitudinal section from an animal that received injection of adjuvant alone. The site of the crush is detected where anterogradely labeled axons stop abruptly. The few axons that extend past the crush are found only along the peripheral

region of the optic nerve. The bottom panel shows longitudinal section from a different control animal from that shown above. Bar, 100  $\mu$ m

**Figure 8.** The region of the crush in an immunized rat. The top panel shows immunoreactivity for glial fibrillary acid protein showing the region of the crush. The bottom panel shows an adjacent section showing some anterogradely labeled axons that extend past the crush in the central region of the optic nerve. Bar, 50  $\mu$ m.

**Figure 9.** Longitudinal sections from an immunized rat. The top, middle and bottom panels show three different sections from the same optic nerve from an animal treated with the immunization procedure. Anterogradely labeled axons that extend past the crush in the central region of the optic nerve can be seen in all three sections. Bar, 100 $\mu$ m.

**Figure 10.** Longitudinal sections from an immunized rat. This section is from a different animal than that shown in Figures 8 and 9. In this animal, axons were observed to extend farther than 1 mm from the site of the crush. Bar, 100  $\mu$ m.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention resides in the discovery that the administration of compositions, comprising immunogenic molecules derived from neuron growth inhibitors, to a mammal cause polyreactive antibodies to be generated that bind to neuron growth inhibitors at the site of neuron damage or disease, thereby promoting axon regeneration. In particular, this type of immunization approach can be used to promote axon regeneration in an injured or diseased human CNS. The compositions can also be used as prophylactic vaccines.

The compositions of the present invention comprise *immunogenic agents* that cause *polyreactive antibodies* to be generated in a subject, which bind to the subject's own neuron growth inhibitory molecules and block their effect, thereby allowing for axon regeneration.



Immunogens correspond to a class of molecules that elicit an immune response through classical immunologic pathways, as in the non-limiting example of the incorporation in an MHC molecule of an antigen processing cell where the immunogens can potentially interact with antigen specific T cell receptors. Alternatively, as another non limiting example, immunogens can bind to antigen specific binding regions of immunoglobulins which may lead to stimulating the B lymphocytes (if on the surface of B lymphocytes), but alternatively could elicit an immune response through other means, e.g., by the activation of complement, or the modulation of Fc receptors.

When multiple immunogens, and/or multiple dosings of the same immunogen, are administered, the individual doses of individual immunogens may by themselves be subimmunogenic, provided that in aggregate, when administered according to the schedule, an immunogenic effect is achieved.

The compositions of the present invention create an immunogenic response by causing *polyreactive antibodies* to be generated in a subject. Polyreactive antibodies are natural autoantibodies that bind to a large variety of structurally unrelated antigens. They are generally of the IgM class. These natural autoantibodies, which are present in the sera of healthy humans and rodents, are encoded by germline genes with no or few mutations. Generally, they recognize long stretches of acidic amino acids and thus have broad antigen binding capabilities. The physiological function of natural autoantibodies is not known, but they may participate in general homeostasis. (Asakura *et al.*, (1995) *Molec. Brain. Res.* 34:283-293)

*Immunogenic agents:*

Any individual proteins shown to have neuron growth inhibitory properties *in vitro* are suitable immunogenic agents of the compositions of the present invention.

The immunogenic agents of the composition are inhibitors of axonal growth, including both myelin-associated inhibitors, such as NI 35/250, MAG, arretin, and the tenascins, and non-myelin-associated inhibitors, including inhibitors present in scar tissue, such as the proteoglycans CSPGs, phosphacan, and versican.

These immunogenic agents may be delivered alone or in combination to stimulate an appropriate polyreactive antibody response to promote axon regeneration.

These immunogenic agents may be mammalian or submammalian, but preferably are human.

Any method of providing a composition of immunogenic agents to a subject may be used. Strategies include the administration of purified growth inhibitor proteins, or fragments thereof, the administration of recombinant growth inhibitor proteins, or fragments thereof, and gene therapy.

Immunogenic agents may be obtained from a natural source; for example, immunogenic agents may be derived from CNS tissue (including spinal cord, brain, brainstem, and optic nerves). In particular, immunogenic agents may be obtained from spinal cord extracts. Immunogenic agents may also be derived from the peripheral nervous system.

Although immunogenic agents may be associated with any component of the CNS tissue, they associate preferentially with myelin components (Mckerracher *et al.*, (1994) *Neuron* 13:229-246 and 805-811). Accordingly, myelin may be purified to develop a more refined composition of immunogenic agents. To further purify the immunogenic agents needed for immunizations, the spinal cord tissue is homogenized in a physiological buffer and subjected to a standard protocol to separate the myelin. The spinal cord is used here because it has a high content of myelin. This procedure, however, can also be done with any other parts of the CNS (such as the brain, brainstem, optic nerves, etc.). The biochemical separation of myelin from the spinal cord homogenate will yield two components - (1) the purified myelin, and (2) the non-myelinated components.

The purified myelin can be subjected to further biochemical separations, such as detergent extractions, followed by various types of separations techniques, including anion or cation exchange chromatography, but not limited exclusively to these forms of separations. The various partially purified myelin components can be used to immunize mice, and the effectiveness of these components to promote axon regeneration assessed.

An immunogenic agent mixture may comprise either whole myelin or partially purified components of myelin. For example, purified myelin-associated axon growth inhibitors, such as NI 35/250, MAG, arretin, or the tenascins, could be used alone or in combination to stimulate an appropriate polyreactive response to promote axon regeneration.

Other growth inhibitors, not primarily associated with myelin could also be used in the composition. In particular, growth inhibitors associated with scar tissue, such as proteoglycans, may be used.

Alternatively, the growth inhibitor proteins, or fragments thereof, may be made by recombinant means utilizing available sequence data. These recombinant proteins could be combined to create a well-defined immunogen composition.

Purified or recombinant proteins could be used alone or in combination in the composition. The composition does not have to contain only previously identified molecules, but could also contain unidentified growth inhibitors.

The composition may be prepared in pharmaceutically acceptable form according to known method steps, based on the teaching and guidance presented herein. Recombinant technology and monoclonal antibodies and fragments thereof may also be used (See, e.g., Ausubel *et al.*, eds, (1987, 1993) *Current Protocols in Molecular Biology* (Greene Publishing Assoc. and Wiley Interscience, N.Y., N.Y.); Coligan *et al.*, eds., (1992, 1993) *Current Protocols in Immunology* (Greene Publishing Assoc. and Wiley Interscience, N.Y., N.Y.); Sanbrook *et al.*, *infra*; and Harlow, *infra*).

Purification of immunogenic agents helps minimize the presence of impurities in a immunization composition. Purification may be performed by any method that produces a highly pure end product as in the non-limiting examples of: chromatography, electrokinetic processes, membrane processes, centrifugation, and extraction (Biotechnology 5:789-793). Extensive reviews of separation techniques are included in several different texts including the book by Juan A. Asenjo,

Separation Process in Biotechnology, published Marcel Dekker Inc., New York (1990) (see especially Chapters 19, 20).

The composition should preferably contain as few impurities as possible, as well as maintain a very high consistency of the amounts and ratio of an immunization composition components including immunogens and adjuvants in each dose with little or no lot-to-lot variation. An immunization composition immunogens should preferably be characterized by their unique physical properties to allow their composition in an immunization composition to be measured. The movement of an immunogen or its immunogenic components through a gel as in the non-limiting examples of chromatography or electrophoresis will allow detection of bands of impurities in the immunization composition. In a preferred embodiment the total amount of impurities comprising molecules unrelated to the desired an immunization composition components should be less than 0.5% by weight and even more preferably less than 0.1%. The desired an immunization composition antigens should preferably be defined by molecular weight and molecules should preferably differ by less than 5% of this number or be considered separate immunogen. The variation in the amount of a particular defined immunogenic composition component, such as an immunogen or adjuvant, should preferably be less than 2% by weight. A non-limiting example of how immunization compositions (eg. vaccines) are currently tested for purity is described in a paper on manufacturing of *Neisseria meningitis* vaccines (Avshalom Mizrahi, Bacterial Vaccines, Alan R. Liss Inc. (1990), pages 123-145) and differences to the current inventions are clear.

#### *Gene Therapy:*

Gene therapy can also be used to provide immunogenic agents to a subject. Gene therapy methods include the use of recombinant viral vectors encoding growth inhibitors.

Generally any virus capable of infection and gene transfer can be employed. Suitable viruses for this invention include adenoviruses, adeno-associated virus, herpes simplex viruses, the AIDS virus, and retroviruses well known to those skilled in the art.

The viral vector employed may, in one embodiment, be an adenoviral vector that includes essentially the complete adenoviral genome (Shenk *et al.*, (1984) *Curr. Topics Microbiol. Immun.* 111(3):1-39). Alternatively, the viral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted. Preferably, the viruses used in the construction of viral vectors are rendered replication-defective to remove the effects of viral replication on the target cells.

Generally, any mammalian growth inhibitor can be employed in the present invention. Preferably, the growth inhibitor is human. The DNA sequences can be either cDNA or genomic DNA. DNA encoding the entire growth inhibitor, or any portion thereof, may be used. Due to the degeneracy of the genetic code, other DNA sequences that encode substantially the same growth inhibitor or a functional equivalent can also be used. Multiple gene copies may also be used.

The sequence of specific growth inhibitor genes have been published. Any other growth inhibitor sequences published in the future can also be used in this invention.

The DNA sequences encoding the growth inhibitor are under the control of a suitable promoter. Any suitable promoter or any portion thereof may be employed to mediate expression, including a growth inhibitor gene's own promoter, other neuron-specific promoters such as NF-H, NSE, Thy-1, or prion, or a viral promoter such as the CMV or SV40 promoter.

In order to produce the gene constructs used in the invention, recombinant DNA and cloning methods, which are well known to those skilled in the art, may be utilized (see Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2d ed. (New York: Cold Spring Harbor Laboratory Press, 1989), including the use of restriction enzymes, site directed mutagenesis, ligation, homologous recombination, and transfection techniques.

It will be appreciated that administration of the viral vectors of the present invention will be by procedures well established in the pharmaceutical arts, e.g. by direct delivery to the target organ, tissue or site, intranasally, intravenously, intramuscularly, subcutaneously, intradermally and through oral administration, either alone or in combination.

Preferably, the viral vectors are administered by injecting vector suspension into various locations of the nervous system, or by injection into nerves, or injection into peripheral tissues such as skin or muscles, which are innervated by neurons. In the latter case, the vector enters the neurons via the axons or axon terminals, and the vector genome is transported retrogradely in the axon to the nucleus.

The dosages administered will vary from subject to subject and will be determined by the level of enhancement of neurofilament function balanced against any risk or deleterious side effects. Monitoring levels of transduction, neurofilament expression and/or the presence or levels of normal neurofilament will assist in selecting and adjusting the dosages administered.

*Compositions:*

In addition to the antigens, the pharmaceutical composition may also contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

Such carriers may include depot adjuvants that release an immunogen in vivo over a prolonged period as compared to administration of an unbound immunogen. Preferably the depot adjuvant comprises an aluminum, calcium or salts thereof, such as aluminum sulfate (alum), aluminum phosphate, calcium phosphate or aluminum hydroxide, see, e.g., Gregoriades, G. et al., *Immunological Adjuvants and Vaccines*, Plenum Press, New York, 1989.

Immunogenic compositions of the present invention may also include suitable solutions for administration, intramuscularly, intravenously, subcutaneously, dermally, orally, mucosally, or rectally or by any other injection, and contain from about 0.001 to 99.999 percent, preferably from about 20 to 75 percent of active component (i.e. the immunogen) together with the excipient. Compositions which can be administered rectally include suppositories.

Preparations of immunogenic compositions for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients, such as suitable adjuvants, which are known in the art. Pharmaceutical compositions

such as tablets and capsules can also be prepared according to routine methods. See, e.g., Berker, supra, Goodman, supra, and Avery, supra, which are entirely incorporated herein by reference, included all references cited therein.

The immunogenic compositions of the present invention may optionally include immunomodulators other than antigens. Such immunomodulators may also be administered separately as a part of the program.

The immunogenic compositions of the present invention may also include pharmaceuticals whose primary activity is non-immunological. Said non vaccine pharmaceutical agent may be of any class of pharmaceutical agents including the non limiting examples of agents to ameliorate the following diseases: infectious, cardiovascular, gastrointestinal, endocrine/hormonal, renal, neurological, psychiatric, muscular, skeletal/orthopedic, hematological, hepatic, pancreatic, metabolic, neoplastic, inflammatory/rheumatic, reproductive, dietary/nutritional, ophthalmologic, otologic, pulmonary/respiratory, dermatologic, allergic, and surgical as in anesthetics. See Physicians Desk Reference, Medical Economics Data Production, Montvale, N.J. (1994); United States Pharmacopeia; European PharmaCopeia; Gilman et al, Goodman and Gilman's The Pharmacological Basis of Therapeutics, Macmillan Publishing Company, New York (1985). Preferably, these pharmaceuticals are ones which, when screened as taught herein, are shown to help reduce the incidence or severity of a chronic immune-mediated disorder, or at least do not worsen the disorder.

The term "pharmaceutically acceptable" has both a legal definition and a clinical definition. The code of federal regulations (21 CFR Part 600) legally defines what make an agent "pharmaceutically acceptable."

An immunogenic agent that meets the legal definition of "pharmaceutically acceptable" must further meet the clinical definition of "pharmaceutically acceptable". A clinical definition of pharmaceutically acceptable, as used herein, requires that said agent has a sufficiently beneficial clinical effect when used in a pharmaceutically acceptable dose.

*Use of Immunogenic Compositions:*

The immunogenic compositions of the present invention could be used to treat patients with acute or chronic spinal cord injuries.

To treat acute lesions, immunizations with an appropriate protocol and antigen will be started soon after the accident.

To treat chronic lesions, immunizations with an appropriate antigen will be started and may be combined with other forms of therapy such as neurotrophin or growth factors to stimulate the growth state of the neurons, and/or surgical resections to remove the scar tissue at the site of CNS lesions, with or without transplantations of suitable materials (such as embryonic tissue transplants, purified non-neuronal cell transplants, inert materials, etc.), to serve as a suitable tissue bridge to span the area that has undergone the resection.

This immunization approach can also be used to treat patients with strokes (particularly those with lesions in the axon tracts, e.g., the internal capsule) to promote regeneration of axons.

This immunization approach can also be used in patients with Multiple Sclerosis, particularly in patients who display signs of axonal damage such as of the optic nerve, or other long axon tracts in the spinal cord or brain.

A modification of this immunization approach can be used as a prophylactic vaccine in healthy individuals. This would prime the immune system such that later immunizations given to an individual who has sustained CNS injury or onset of CNS disease (such as MS or stroke) would provoke a more rapid and strong antibody production to stimulate regeneration of axons.

*Immunization protocol:*

The following immunization protocol is used to preferentially result in the production of a polyreactive autoantibody response. Variations of this protocol that results in the production of polyreactive antibodies to axon growth inhibitors to be used as a therapy in humans would also be included in this invention.



While it is possible that the purposes of the present invention can be served with a single administration, especially when the immunogen is a strong one, and in that case a single dosing schedule is within the compass of the present invention, it is desirable to administer two or more dosings for greater surety. Preferably, the number of dosings is at least three, more preferably at least four, and still more preferably, at least five. There is no set maximum number of vaccinations, however it is good clinical practice not to immunize more often than necessary to achieve the desired effect.

It will be appreciated by those skilled in the art that a variety of possible combinations and subcombinations of the various preferred conditions of timing of the first administration, shortest interval, largest interval and total number of administrations (in absolute terms, or within a stated period) exist, and all of these combinations and subcombinations should be considered to be within the inventor's contemplation though not explicitly enumerated here.

There are a number of assays that can be used to determine immune system response. *In vivo* assays include the non-limiting examples of antibody responses and delayed type hypersensitivity responses. The antibody responses primarily measures B-cell function as well as B-cell T-cell interactions while the delayed type hypersensitivity responses measure T-cell immunity. One may compare antibody titers in the blood following an antigenic challenge. These levels may be quantitated according to the type of antibody as in the non-limiting examples of IgG, IgG1, IgG2, IgM, or IgD. One can also compare development of immune system response by determining levels levels of antibodies and lymphocytes in the blood without antigenic stimulation.

Phenotypic cell assays can also be performed to determine the frequency of certain cell types. Peripheral blood cell counts may be performed to determine the number of lymphocytes or macrophages in the blood. Antibodies may be used to screen peripheral blood lymphocytes to determine the percent of cells expressing a certain antigen as in the non-limiting example of determining CD4 cell counts and CD4/CD8 ratios.

*Dose:*

A pharmaceutically acceptable dose, as in total dose, is a dose where the clinical benefits of said product outweighs the toxicity at said dose. Non-limiting examples of said toxicity include acute or subacute reactions like fever, shock or seizures, which may lead to permanent sequela and chronic toxicity like cancer, as is known and recognized in the relevant parts. A pharmaceutically acceptable dose according to this definition can vary according to the severity of the illness being modulated by the immunogenic agent. It is logical that a high dose of an agent which causes significant toxicity may be pharmaceutically acceptable in certain situations.

A pharmaceutically acceptable dose will depend on the structure of the particular agent and/or the condition of the recipient. Some reagents may be more toxic than others while some may be more immunogenic than others. In a like manner, some individuals may be more responsive to a given dose while others may be more sensitive to the toxic effects at that dose. There is thus an individual variation within the definition of pharmaceutically acceptable dose as well as species, racial, age, and population variation, all of which should be taken into account when dosing an individual. Such consideration has, of course, been given to other prophylactic agents.

*Assessing the Efficacy of Immunogenic Agents:*

One animal model that can be used to assess the effectiveness of immunogenic agents is spinal cord injury in the adult mouse.

The protein levels of immunogenic agents are monitored by observing optical density (OD) measurements. The mouse is then immunized with a known amount of protein (50µg, for example) in Incomplete Freund's adjuvant per mouse, subcutaneously 2 times weekly for 3 weeks.

The dorsal portion of the spinal cord in the lower thoracic region is lesioned with microscissors (hemisection) as described by Li *et al.*, (1996) *J. Neurosci. Res.* 46:404-414, and the immunization continued for another 3 weeks post-lesion period in the same way as above. After this 3 week period, Wheatgerm conjugated horseradish peroxidase (WGA-HRP) is injected into the sensory-motor cortex. After 24 hours, blood is collected for testing and the animals are perfused with fixatives for HRP histochemistry. The protocol used for the HRP histochemistry

*in vivo* assay of regeneration will be similar to that described (Li *et al.*, (1996) *J. Neurosci. Res.* 46:404-414).

Additionally, retrograde neuronal tracers can be used to assess regeneration. This is done by applying a fluorescent dye such as Fluorogold to the site of the spinal cord lesion at the time of lesioning. After 3 weeks post-lesion, during which the animals receive immunizations, the animals will receive a 1 µl injection of a second fluorescent dye such as Fluororuby at a point between 5-6 mm caudal to the site of the spinal cord lesion. Three days later the animals are sacrificed by perfusion with a fixative such as 4% paraformaldehyde and the brain cut on a cryostat through the regions containing the sensory-motor cortex.

At the time of sacrifice, the serum of the animals is collected and tested in an *in vitro* neurite growth assay as described previously by Li *et al.*, (1996) *J. Neurosci. Res.* 46:404-414, and also used for biochemical studies such as Western blotting, ELISA and immunohistochemistry using standard protocols.

While not wanting to be bound by any theory, it is proposed that the antibodies likely enter into the degenerating fiber pathways in the white matter as the blood-brain barrier is compromised due to disease, injury, or trauma. We have demonstrated that not only does the immunization procedure lead to increased levels of circulating myelin-reactive antibodies but that these antibodies cross the blood-brain barrier and are bound to myelin in the white matter of the hemisected spinal cord. Previous studies have shown that contusion injury to the adult rat spinal cord results in an increase in the permeability of the blood-spinal cord barrier for about 30 mm on either side of the lesion (Popovich, P.G. *et al.*, *Exp. Neurol.* 142, 258-275 (1996)). The same study also showed a secondary increase in blood-spinal cord permeability specifically in the white matter at 14-28 days post-injury (Popovich, P.G. *et al.*, *Exp. Neurol.* 142, 258-275 (1996)). In addition to the acute break-down of the blood-brain/spinal cord barrier immediately after injury, at later stages this barrier may be opened by the action of microglia (Popovich, P.G. *et al.*, *Exp. Neurol.* 142, 258-275 (1996); Jensen, M.B. *et al.*, *Exp. Neurol.* 143, 103-116 (1997)). Therefore, the myelin-reactive antibodies which are produced by our immunization procedure are likely to enter into the degenerating fiber pathways in the white matter as the blood-brain barrier

is opened by the trauma and the action of activated microglia. These antibodies then bind to inhibitors associated with myelin and thus stimulate axon regeneration over long distances.

We did not see any evidence of cellular inflammatory changes in the spinal cord similar to that seen in EAE. Induction of EAE in most rodents requires immunization with myelin proteins mixed with complete Freund's adjuvant often along with pertussis toxin (Traugott, U., *et al.*, Cellular Immunology 91, 240-254 (1985); Goverman, J., *et al.*, Critical Rev. Immunol. 17, 469-480 (1997)). In fact, pretreatment of mice with myelin or myelin proteins in IFA has been shown to protect animals from EAE (O'Neill, J.K., *et al.*, J. Neuroimmunol. 41, 177-187 (1992)); Tonegawa, S.M.S., *et al.*, J. Exptl. Med. 186, 507-517 (1997); Rivero, V. E., *et al.*, J. Neuroimmunol. 72, 3-10 (1997)), which is a T-cell mediated disease. Protection by this and related types of treatment is thought to be due to clonal deletion and immune deviation of autoreactive T-cells (Amitabh, G., *et al.*, Science 258, 1491-1494 (1992); Weiner, L.H., *et al.*, Annu. Rev. Immunol. 12, 809-837 (1994)).

*Advantages of the Invention:*

Some advantages of this invention are as follows: (1) Several, if not all, of the axon growth inhibitors can be blocked at the same time. (2) It is not necessary to insert antibody-producing hybridoma cells (which produce monoclonal antibodies) into the CNS, or infuse purified antibodies via osmotic pumps. The transplantation of hybridoma cell lines could lead to tumors that would ultimately have detrimental side effects in humans. (3) With this approach it is not necessary to infuse antibodies or FAB fragments of an antibody; rather, the approach of the present invention is to manipulate the animal or patient's own immune system to produce polyreactive antibodies that can block axon growth inhibition by myelin or other components of the white matter such as astrocytes.

This approach to stimulate regeneration in the CNS is useful therapy to be applied after spinal cord injuries, other types of CNS traumas, strokes, multiple sclerosis, optic nerve damage, and other neurological conditions in which axon regeneration is desired.

This approach is particularly useful because one could start immunizing a person at the scene of an accident involving spinal cord injury, or very soon thereafter; thus, there would be an immediate production of polyreactive autoantibodies and the corresponding stimulation of axon regeneration. This treatment could be combined with other types of therapies such as growth factor or neurotrophin therapies, or methylprednisolone treatments.

The compositions of the present invention could also be used as prophylactic vaccines to be given to healthy individuals. Commencement of immunization after the onset of CNS lesion or disease (such as a stroke) would then lead to more rapid and stronger antibody production.

### EXAMPLES

The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

The following examples demonstrate that damaged axons in the spinal cord and optic nerve regenerate for long distances in animals immunized with this protocol. One set of experiments tests regeneration of axons in the spinal cord of adult mice. A second set of experiments tests regeneration in the injured optic nerve of adult rats.

A homogenate of the spinal cord from a normal mammal was used to generate polyreactive antibodies in the same species of mammals (Miller *et al.*, (1994) *J. Neurosci.* 14:630-6238). In particular, these examples demonstrate that the production of polyreactive antibodies in animals stimulates the regeneration of damaged axons. Furthermore, these examples show that this treatment results in recovery of motor function after spinal cord lesions.

## **EXAMPLE I**

### **Preparation of Antigens**

#### *Preparation of myelin:*

Normal mice (4-8 weeks old) are deeply anaesthetized with chloral hydrate, and perfused via the heart with 0.1M phosphate buffer. Brains and spinal cords are removed and collected separately in buffer containing protease inhibitors.

Purified myelin is prepared by the procedure of Norton and Poduslo (1973), with the inclusion of protease inhibitors at every step. Briefly, this consists of homogenizing the tissue in 0.32 M sucrose containing protease inhibitors, with a motor-driven Dounce homogenizer. The homogenate is then filtered through a cheese cloth and then overlaid on 0.85 M sucrose and centrifuged for 30 min at 75,000g. The material at the interface is collected and resuspended in ice cold water and centrifuged for 25 min at 25,000 g. The pellet is again resuspended in the same way and centrifuged for 15 min at 10,000 g. This step is repeated. The pellet is then resuspended in 0.32 M sucrose, overlaid on 0.85 M sucrose, and centrifuged for 1 h at 75,000 g. Purified myelin, which separates at the interface, is collected and washed twice with ice cold water.

#### *Anion-exchange chromatography:*

Purified myelin is extracted for 2 hr at 20EC with 1% octylglucoside (1ml per milligram of protein) in 0.2 M phosphate buffer (pH 6.8) containing 0.1 M Na<sub>2</sub>SO<sub>4</sub>, 1mM EDTA, 1mM dithiothreitol, and a composition of protease inhibitors. The extracts are centrifuged at 400,000 g.min and applied to a DEAE-Sepharose column (Pharmacia; 1 cm x 1cm). After several washes, elution is effected with a NaCl gradient (0.2-2 M) containing 1% octylglucoside, 50 mM Tris-HCl (pH 7), and 1 mM dithiothreitol. Fractions are collected and protein concentrations estimated.

## **EXAMPLE II:**

### ***In Vitro* Assay for Testing the Efficacy of the Immunogenic Composition**

**to Stimulate Neurite Growth on a CNS Inhibitory Substrate**

The efficacy of the immunogenic composition of the present invention was assessed by measuring neurite growth *in vitro*, using both NG108-15 cells and primary neurons. Neurite growth was assessed using a previously-described assay (Mckerracher *et al.*, (1994) *Neuron* 13:229-246 and 805-811).

*NG108-15 cells:*

Purified myelin (8 µg/ well) or the appropriate antigen (e.g., purified inhibitory components used for immunization) was plated onto poly-L-lysine (PLL)-coated 96 well plates and left overnight. The wells were then washed with buffer. Cyclic AMP primed NG108-15 cells were first labeled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, 3 µg/ml) in serum-free tissue culture medium, then rinsed and maintained in culture for 24 h prior to use in the assay. These cells were added to the wells at a density of  $10^3$  cells per well, and cultured for 24 h in Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum and 1mM dibutyryl cAMP. The cultures were then fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer for 30 min.

Alternatively, the unlabeled NG108-15 cells were plated and used in the neurite growth assay as noted above. At the end of the experiment, however, these cells were fixed as described above and stained with Coomassie blue for 5 min.

Neurite growth was assessed by estimating the percentage of cells extending neurites greater than 1 cell body diameter in length using an inverted microscope equipped with fluorescence optics. Counts were made from duplicate wells in each experiment.

A similar assay was done using primary neurons. Embryonic day 19 rat hippocampal neurons were prepared as described previously (Banker and Cowan, 1977). Neonatal rat cerebellar granule cells were purified by Percoll density gradient centrifugation as described by Hatten (1985). These neurons were then plated onto round glass coverslips coated with the immunogenic

composition. After 18-20 hrs, the cultures were fixed and labeled with a monoclonal antibody against GAP-43 and visualized with a goat anti-mouse Ig conjugated to rhodamine.

### EXAMPLE III

#### Axon Regeneration After Spinal Cord Injury

The following example illustrates that immunization with the immunogenic composition stimulated axon regeneration in a mammalian subject following spinal cord injury.

##### *Immunization of mice:*

Adult Balb/c mice were immunized subcutaneously twice weekly with an immunogenic composition. The composition consisted of 50µg of either adult mouse spinal cord or purified CNS myelin homogenized in phosphate buffered saline and mixed with an equal volume of incomplete Freund 's adjuvant.

##### *Spinal lesions:*

After 3 weeks of immunization, the animals were anesthetized. The mid to lower thoracic spinal cord was exposed and a dorsal hemisection lesion done with a pair of microscissors. The lesions were carried out in such as way as to cut both corticospinal tracts that run from the motor cortex to various spinal cord levels. The area was closed by sutures, and the animals were allowed to survive for another three weeks. The animals continued to receive twice weekly immunizations during this 3 week period.

##### *Histochemistry:*

At the end of three weeks post-lesion, the animals were anesthetized. Wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP), a neuronal tracer for axon regeneration, was micro-injected into the motor cortex. Twenty-four hours later, the animals were sacrificed and the spinal cord removed for cryostat sectioning and HRP histochemistry. Thirteen animals were examined with HRP. All of these showed excellent HRP-labeling of the corticospinal tract axons proximal to the injury. In seven animals (54%), large numbers of HRP-labeled axons were seen



extending past the lesion into the distal portion of the spinal cord, suggesting that these axons had regenerated past the lesion. (Fig. 1a, b, c, d, e) To confirm that the axons were lesioned, we counterstained the tissue sections with Nuclear Red. This staining of the sections clearly showed increased cellularity at the site of the lesion, suggesting the presence of a scar; thus, the axons are likely to have been injured. Control mice injected with incomplete Freund's adjuvant alone and lesioned in the same manner did not show any labeled axons distal to the lesion.

These results clearly indicate that immunizing animals with an appropriate antigen to produce a polyreactive antibody response generates sufficient circulating antibodies to block inhibitors of axon growth and thus stimulate axon regeneration.

*Retrograde Neuronal Labeling:*

To confirm that the large numbers of WGA-HRP labeled axons that crossed the lesion in the immunized mice were indeed lesioned axons that had regenerated and not ones that might have been spared, retrograde double-labeling studies were carried out.

Since myelin-associated inhibitors have been reported to play a crucial role in the failure of CNS regeneration (Schnell & Schwab (1990) *Nature* 343:269-272; Bregman *et al.*, (1995) *Nature* 378: 498-501), we compared the effects of spinal cord homogenates (n=6) to myelin purified from either mouse (n=12) or bovine (n=4) CNS. The immunization and lesioning protocols were as described above. The retrograde tracer Fluorogold (Schmued & Fallon (1986) *Brain Research* 377:147-154; Novikova *et al.*, (1997) *J. Neurosci. Meth.* 74:9-15) was applied at the site of lesion to label cut corticospinal tract axons. Our studies with the unlesioned corticospinal tract (results not shown), as well as previous work (Schmued & Fallon (1986) *Brain Research* 377:147-154) indicated that this marker is taken up only by damaged axons. After three weeks, a second tracer, either Fluororuby (Novikova *et al.*, (1997) *J. Neurosci. Meth.* 74:9-15) or cholera toxin subunit B (Tavares *et al.*, (1996) *J. Comp. Neurol.* 374:84-95), was injected into a site about 5-6 mm caudal to the lesion. Animals were sacrificed one week later and cryostat sections of the motor cortex examined for the presence of double-labeled neurons.

As with the WGA-HRP labeling studies, 55% of the mice immunized with spinal cord homogenate or purified myelin showed good evidence of regeneration of corticospinal tract axons as revealed by the presence of double-labeled neurons in the sensory-motor cortex (Figs. 1f, 1g, 2). In mice that demonstrated strong evidence of axon regeneration, 50% " 16% of the motor neurons were double-labeled (i.e., regenerated neurons). In some mice, about 75% of the neurons were double-labeled (Fig. 2). Since not all of the Fluorogold-labeled neurons project to the site of the second injection, the number of double-labeled cells represents a substantial number of regenerating neurons. Only 6% " 5% of the motor neurons in immunized mice were labeled with the second marker alone, representing unlesioned or spared neurons. In the majority of these animals (60%), less than 1% of the neurons were spared. Double-labeled neurons were rarely seen (0.19%) in control animals with spinal cord hemisection (n=9) (Fig. 2).

These results provide strong evidence that injured corticospinal tract axons regenerate in mice immunized with a homogenate of spinal cord or purified CNS myelin in IFA.

#### *Assessment of Motor Control:*

Recovery of motor control of the foot and toes was assessed three weeks after lesioning in a group of mice immunized with spinal cord homogenate (Figure 3). Controls were injected with IFA only. The following tests were used: (1) spreading and closing of the toes, and (2) stepping of the hind-limb in response to stroking of the dorsum of the foot. About 66% (n=12) of the treated animals were able to spread and close the toes of the hind limbs as compared to only 10% (n=10) of control mice. Stepping with the hind-limbs after stroking of the dorsum of the foot was detected in 58% of the immunized mice but in none of the control mice. These results suggest that anatomical regeneration leads to functional recovery as well. The slightly higher percentage of the latter may be due to sprouting from unlesioned descending tracts as previously shown.

#### *In vitro analysis:*

Additionally, the serum from the immunized mice was tested in an *in vitro* neurite growth assay (described above) for their ability to block the inhibitory activity of myelin. The serum of three of the animals that showed axon regeneration *in vivo* were tested and compared to the serum of an uninjected normal control mouse. The sera of the injected mice were able to stimulate neurite

growth on the inhibitory myelin substrate as compared to control serum. These results indicate that the antibodies in the serum of the immunized mice were able to block the inhibitors found in the myelin substrate.

*Assessment of myelin-reactive antibodies:*

In addition, at the time of sacrifice, the serum of the mice was also used for biochemical studies such as Western blotting, ELISA, and immunohistochemistry.

Higher levels of IgG and IgM were present in the serum of immunized mice than in controls (Fig. 4a & b). Serum immunoglobulin in immunized mice reacted with myelin and spinal cord proteins as detected by ELISA (Fig. 4c) and Western blotting (Fig. 5a). Immunoglobulin was also detected by immunofluorescence in the lesioned spinal cord of both immunized and control mice. The staining in immunized mice, however, was much stronger and extended for far longer distances (5 mm) on either side of the lesion as compared to controls when examined 3 days after hemisection. Furthermore, immunoglobulin staining in immunized mice was especially prominent along the dorsal white matter where it appeared to be localized to myelin (Fig. 5b & c). The immunization protocol that we used resulted in the production of antibodies without stimulating inflammatory changes in the CNS. There was no evidence of infiltration of inflammatory cells or demyelination similar to that seen with experimental allergic encephalomyelitis (EAE) in tissue sections of the spinal cord of immunized mice (Fig. 5 d & e).

#### **EXAMPLE IV**

##### **Axon Regeneration in the Optic Nerve**

Another animal model that can be used to assess the effectiveness of the immunogenic agents is an optic nerve lesion in rat. Recently, it has been shown that microlesions in the CNS reduce the extent of the glial scar and allow axons access to CNS white matter distal to the lesion (Davies *et al.*, (1997) *Nature* 390:680-683). Microlesions of optic nerve were therefore used to axotomize RGC axons (Fig. 6). Regenerating RGC axons were visualized by anterograde labeling with cholera toxin and immunofluorescent detection of the cholera protein in longitudinal cryostat

sections of the optic nerve. Some sections were double-labeled with glial fibrillary acidic protein (GFAP) to better resolve the lesion site.

*Preparation of Immunogenic Composition:*

A spinal cord homogenate was made from 2 adult Sprague Dawley rat spinal cords. The spinal cords were removed from adult rats, frozen in liquid nitrogen, and pulverized into powder with a mortar and pestal. Twenty ml of PBS was added to the spinal cord powder, the solution was homogenized with a Dounce teflon-in-glass homogenizer, and the protein concentration was determined by protein assay (Biorad kit). The protein concentration was adjusted to 1.5 mg/ml.

*Immunization of rats:*

Each rat was injected with 150  $\mu$ l of this spinal cord homogenate mixed with 150  $\mu$ l Freund=s incomplete adjuvant. Each injection was performed subcutaneously, twice per week. The rats (n=10) were injected 7 times before optic nerve injury, and twice a week for a further 2 weeks after optic nerve injury. As controls, incomplete Freund=s adjuvant alone was injected (n= 10 animals). A further set of controls received optic nerve injury without injection (n=3).

*Immunochemistry:*

At the time of sacrifice of the animals, the spleens were fixed and examined for enlargement. Also, the blood was collected from rats receiving injection of spinal cord homogenate with adjuvant or adjuvant alone. The blood was left overnight in the refrigerator, and the serum separated from the red cells by centrifugation.

Immunoreactivity of the serum to spinal cord homogenate, myelin protein, or MAG was tested by ELISA. For this, spinal cord homogenate, myelin, or MAG was dried overnight into 96 well plates. Serum diluted 1:10 was added to the plates, and the plates were incubated for 2 hours at 37EC. The plates were washed and treated with anti-rat antibody conjugated with alkaline phosphatase. A color reaction was obtained using 1 tablet p nitropheny phosphate (Sigma) made up fresh in substrate solution (6.4 ml 0.2M sodium carbonate, 18.5 ml 0.2M sodium bicarbonate, 0.2 ml 1 M MgCl<sub>2</sub>, made up to 100 ml). After development of color, the reaction was stopped

with 50  $\mu$ l 3 M NaOH added to a well containing 100  $\mu$ l of substrate solution. The optical density was read with an ELISA plate reader.

*Optic nerve lesion:*

Rats were anesthetized with 0.6 ml/kg hypnorm, 2.5 mg/kg diazepam, and 35 mg/kg ketamin. The left optic nerve was exposed and the sheath was slit longitudinally to allow a 10.0 suture to be passed around the nerve separately from the sheath. The optic nerve was crushed 1 mm from the globe by constriction for 60 seconds (Fig. 6).

*Labeling of axons:*

Three hours before sacrificing the animals, 5  $\mu$ l of 1% cholera toxin  $\beta$  subunit solution (List Biological laboratories, Inc., Cambell, CA) was injected into the vitreous to anterogradely label RGC axons. Two weeks after optic nerve crush the animals were fixed by perfusion with 4% paraformaldehyde, and the eye with attached optic nerve was removed and postfixed in 4% paraformaldehyde. Longitudinal cryostat sections were processed for immunoreactivity to cholera toxin with goat anti-cholera toxin (Ctn) at 1:20,000 (List Biol. Labs Inc, CA), followed by rabbit anti-goat biotinylated antibody (1:200, Vector Labs, Burlingame, CA), and DTAF-streptavidin (1: 500, Jackson Immunoresearch Laboratories). Some sections were labeled with anti-GFAP (1:1000; Dako). Observations of the optic nerve were with a Zeiss Axiomat fluorescent microscope.

Retrograde labeling of RGCs from the superior colliculus (not shown), and anterograde labeling (eg., Ctn) verified that RGC axons were axotomized by the microlesion.

Comparison of control optic nerves to those treated with the immunogenic composition revealed axon regeneration in the latter group (Figs. 7, 8, 9, 10). Numerous axons were observed to extend long distances of over 1 mm in the optic nerve. These axons could be easily identified as regenerating axons by their twisted path of growth, and by the identification of growth cones at the distal end of these fibers. These results demonstrate conclusively that vaccination to produce polyreactive antibodies can stimulate growth and regeneration of retinal ganglion cells in the adult mammalian optic nerve.

In control optic nerves that received a crush alone, no RGC axons extended past the lesion (n=3 animals). In control animals treated with incomplete Freund's adjuvant alone, the crush site was easily detected where most anterogradely labeled axons stopped abruptly. The few axons that succeeded in extending past the crush were typically located at the peripheral aspect of the optic nerve.

Our observations of microlesioned optic nerves treated by a vaccination protocol provide the first evidence that a relatively non-invasive vaccination procedure can help to foster regeneration after injury.

We claim:

1. A composition comprising immunogenic molecules derived from neuron growth inhibitory molecules, which upon administration to a mammal, causes polyreactive antibodies to be generated that bind to axon growth inhibitors, thereby promoting axon regeneration.
2. The composition according to claim 1, wherein the immunogenic agents are derived from mammalian central nervous system tissue homogenate.
3. The composition according claim 2, wherein the central nervous system tissue comprises one or more components of the group comprising: spinal cord, brain, brainstem, optic nerves.
4. The composition according to claim 3, wherein the tissue is largely myelin.
5. The composition according to claim 3, wherein the mammalian origin is human.
6. The composition according to claim 1, wherein the immunogenic agents are derived from mammalian peripheral nervous system tissue homogenate.
7. The composition according to claim 1, wherein said immunogenic agents comprise purified MAG, arretin, proteoglycan proteins.
8. The composition according to claim 3, wherein said proteins are recombinant proteins.
9. The composition according to claim 3, wherein said proteins are purified proteins.
10. The composition according to claim 1, further comprising a pharmaceutically acceptable carrier.

11. A kit comprising the composition of as in claim 10, and instructions for use in immunization and neuron regrowth.
12. A method of promoting axon repair and/or regeneration in a subject by blocking axon growth inhibitors, comprising immunizing said subject with therapeutically effective amounts of an immunogenic composition of claim 1.
13. A use of an immunogenic composition to immunize a mammalian CNS to stimulate production of polyreactive antibodies promote axon repair and/or regeneration.
14. A use of an immunogenic composition as a prophylatic vaccine..
15. The use as in claim 13, wherein the mammal is human.
16. The use as in claim 13, wherein the subject requires axonal repair and/or regeneration due to injury.
17. The use as in claim 13, wherein the injury is acute.
18. The use as in claim 13, wherein the injury is chronic.
19. The use as in claim 13, wherein the injury is selected from the group consisting of spinal cord injuries, optic nerve injuries, and injuries to other white matter tracts.
20. The use as in claim 13, wherein the injury is selected from the group consisting of spinal cord injuries, optic nerve injuries, and injuries to other white matter tracts.
21. The use as in claim 13, wherein the subject requires axonal repair and/or regeneration due to disease.



22. The use as in claim 13, wherein the disease is selected from the group consisting of stroke, multiple sclerosis, and diseases of the optic nerve.
23. The use according to claim 13, further comprising the use of other known therapies comprising cell or tissue transplants, surgical resections to remove scar tissue, neurotrophin or growth factors to stimulate the growth state of neurons.

Figure 1

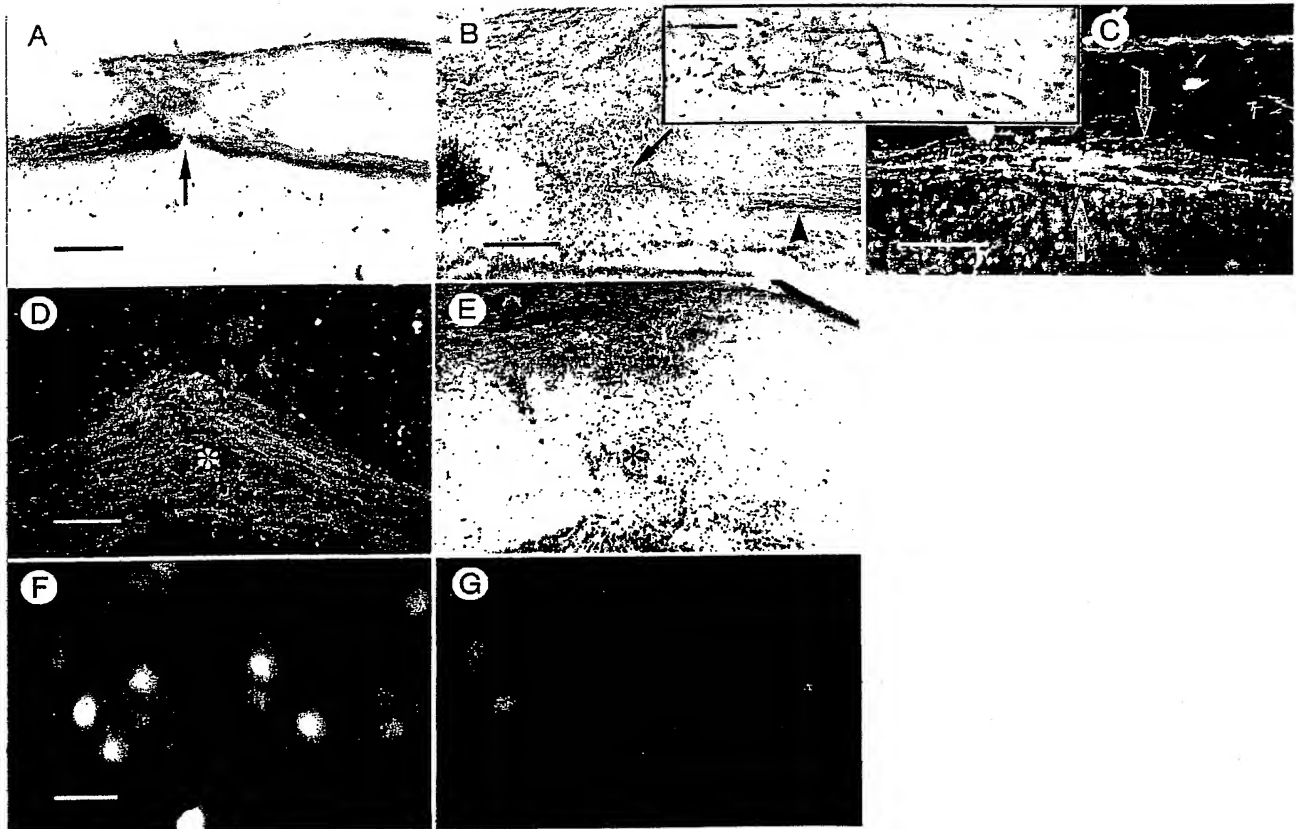
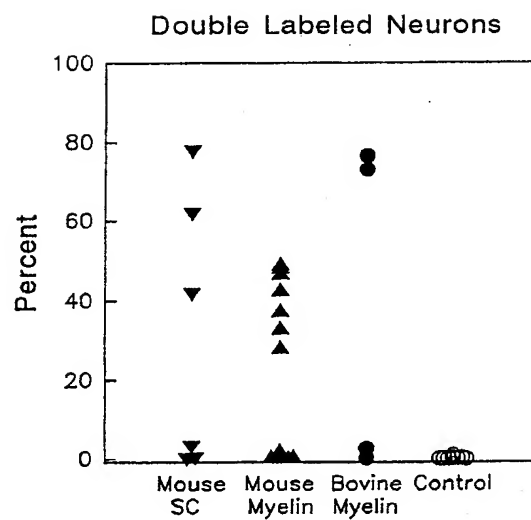


Figure 2



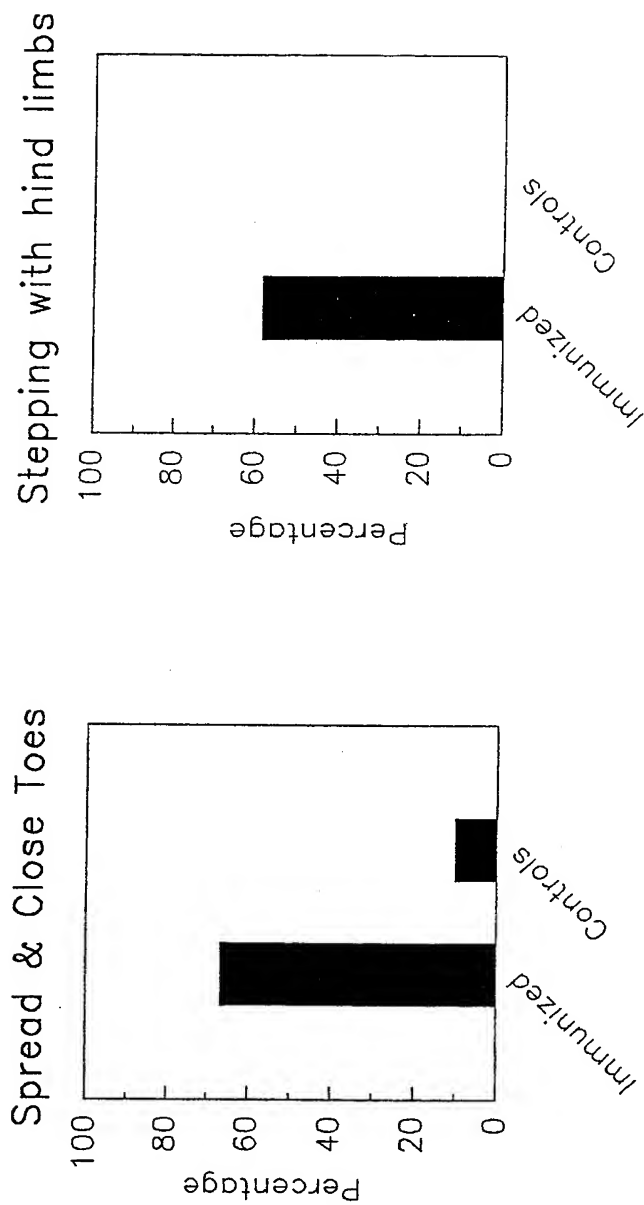


Figure 3

Figure 4

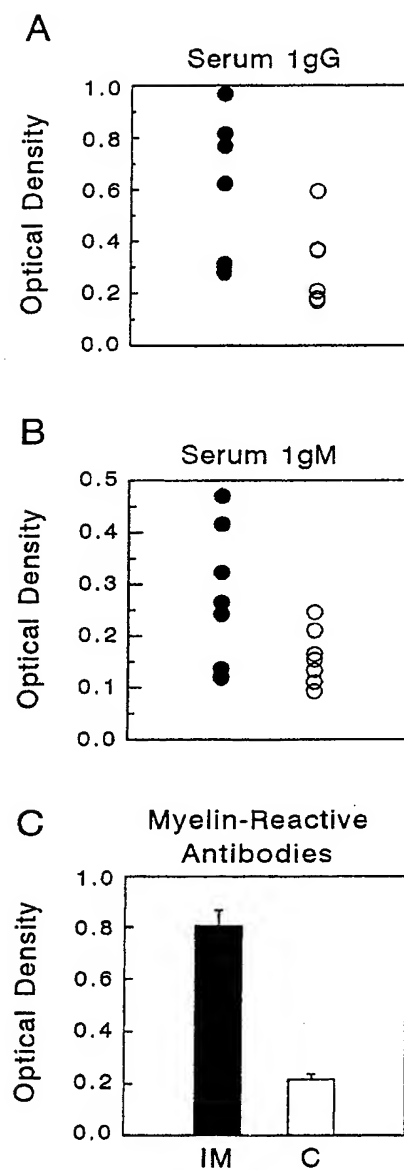


Figure 5

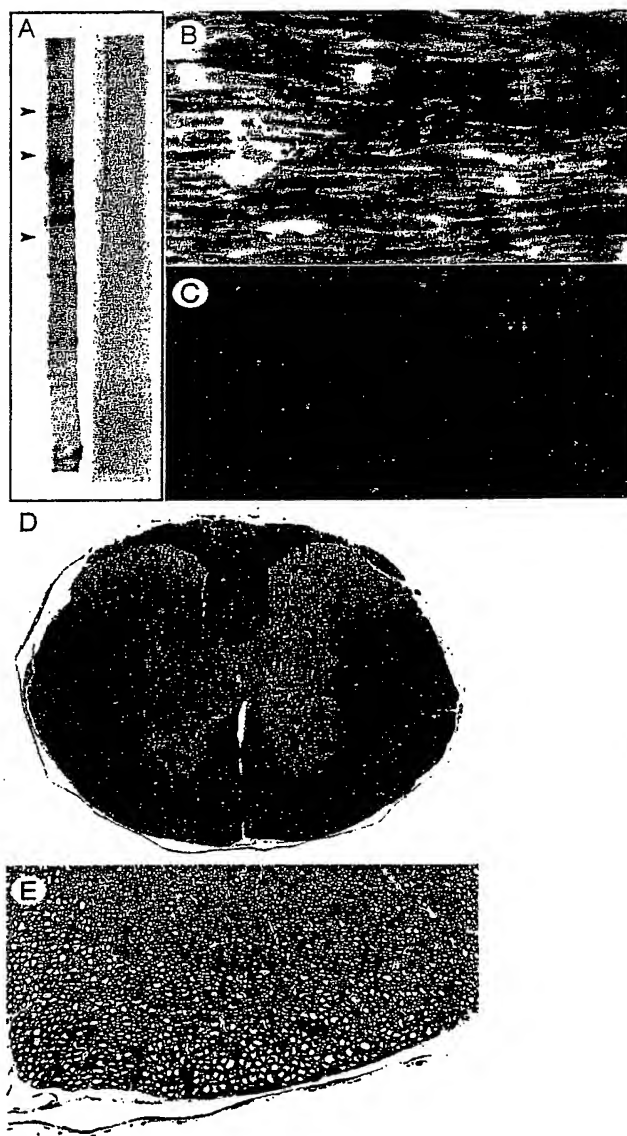
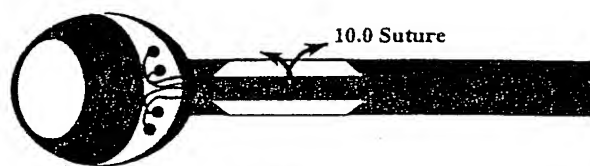


Figure 6

a. Crush



b. Treatment



c. Anterograde Labelling

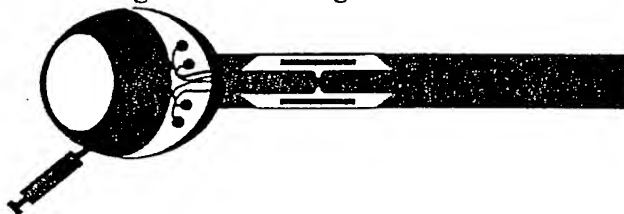


Figure 7

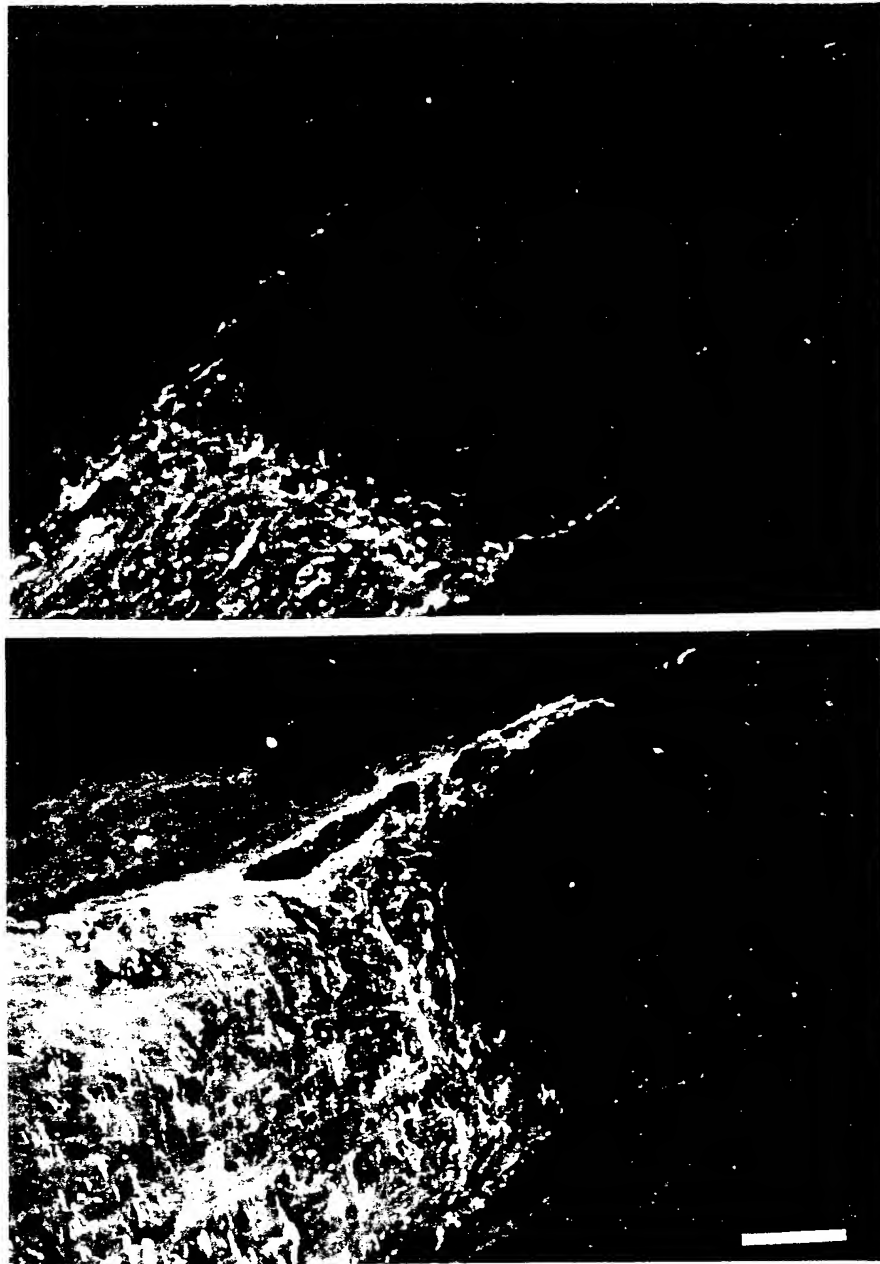




Figure 8

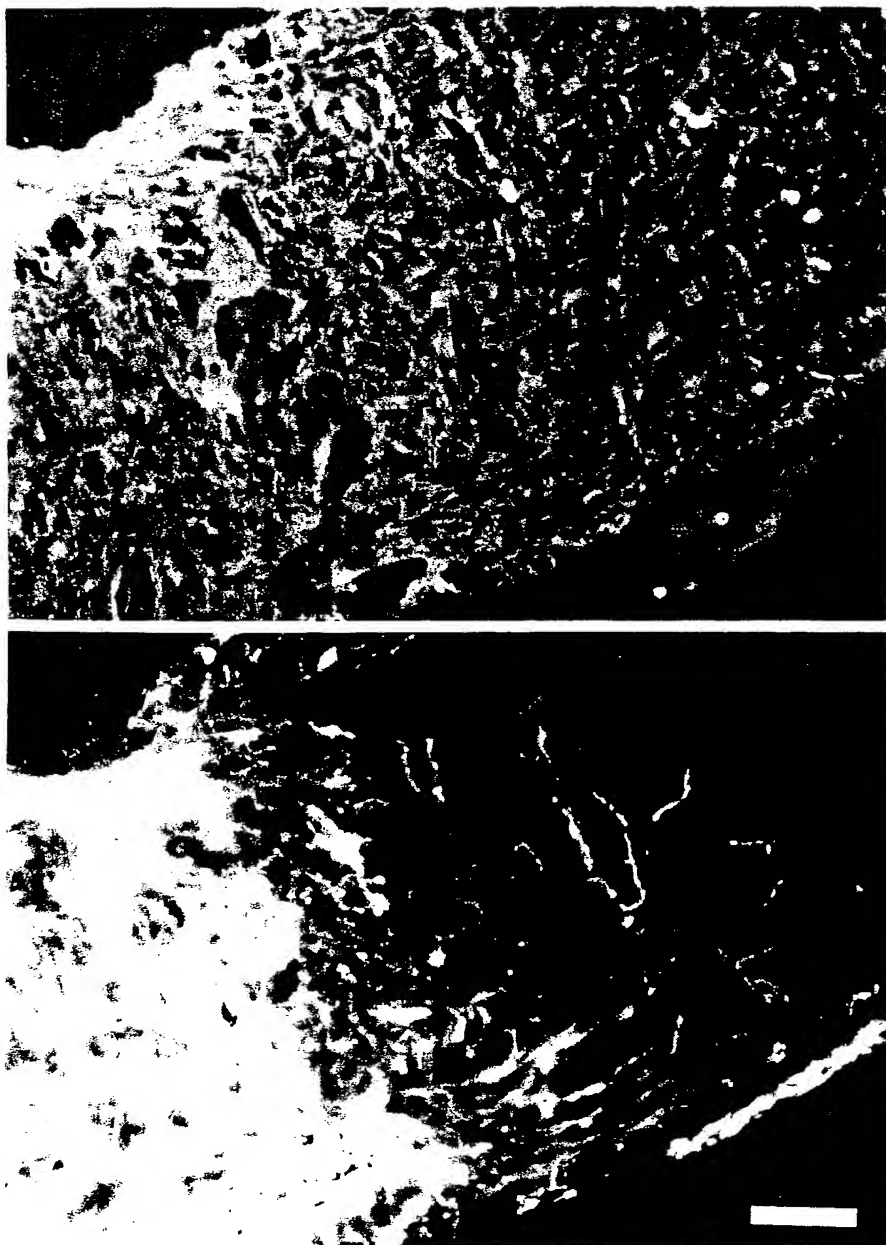


Figure 9

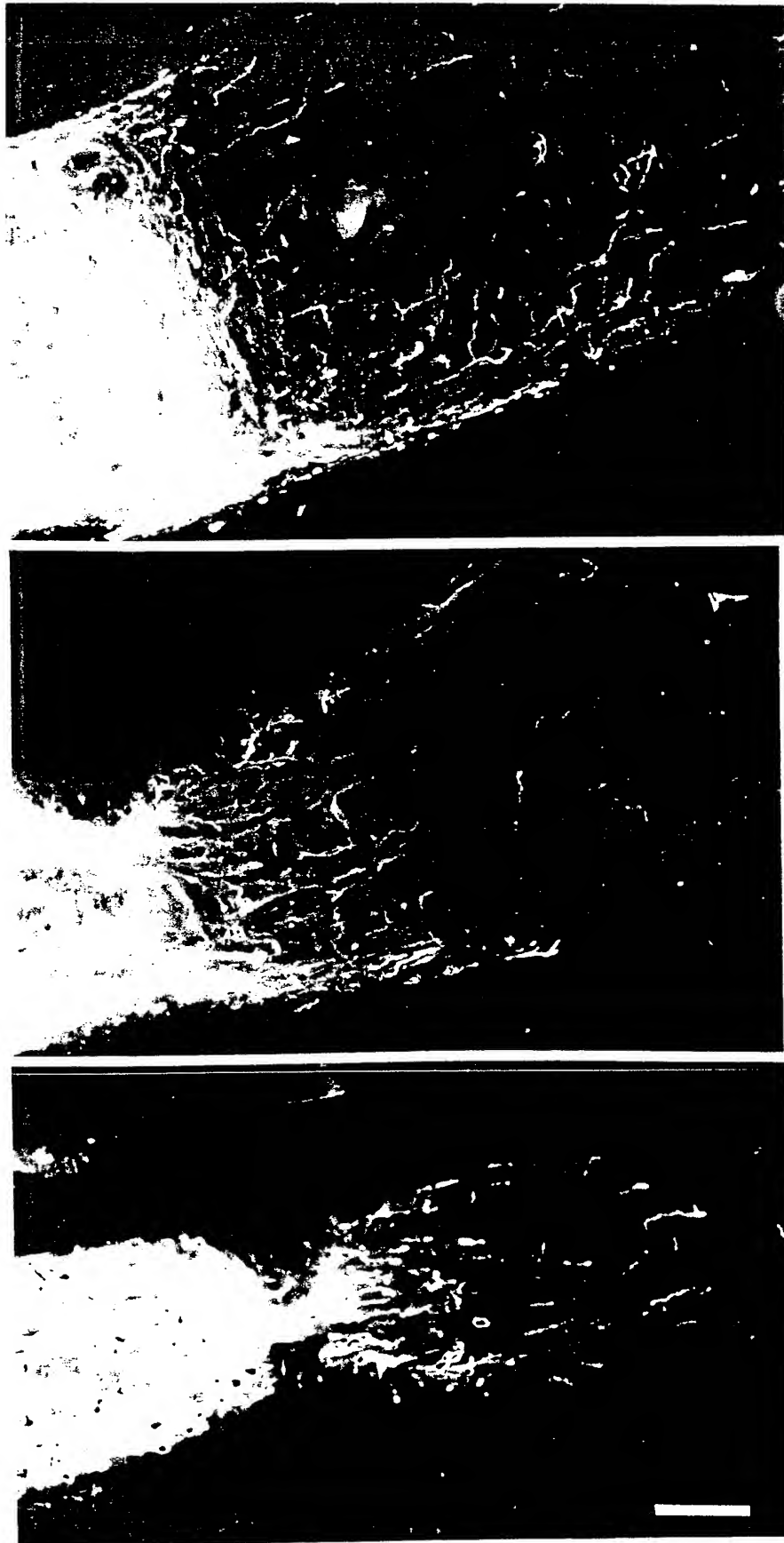


Figure 10



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00304

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 22344 A (MCKERRACHER L. ET AL) 24 August 1995 (1995-08-24) page 2, line 17 - page 5, line 22; claims 2-7 ---	1-23
X	WO 97 07810 A (THE JOHN HOPKINS UNIVERSITY SCHOOL OF MEDICINE) 6 March 1997 (1997-03-06) page 3 - page 4; page 25, line 1; page 32, line 24 - page 33, line 3 claim 1 ---	1-23
X	GB 26535 A A.D. 1913 (NICOLLE C) 14 May 1914 (1914-05-14) the whole document --- -/-	14



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

28 July 1999

Date of mailing of the international search report

30/08/1999

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Le Flao, K

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00304

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI M ET AL: "Myelin - associated glycoprotein inhibits neurite/axon growth and causes growth cone collapse." JOURNAL OF NEUROSCIENCE RESEARCH, vol. 46, no. 4, 15 November 1996 (1996-11-15), pages 404-14, XP002110408 cited in the application page 413, left-hand column, line 23 - line 36	1-23
P,X	----- WO 98 22499 A (MCKERRACHER L. ET AL) 28 May 1998 (1998-05-28) the whole document -----	1-23

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 99/ 00304

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 12-23  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 99/00304

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522344 A	24-08-1995	AU 1703595 A CA 2183630 A	04-09-1995 24-08-1995
WO 9707810 A	06-03-1997	AU 6858596 A	19-03-1997
GB N26535 A		NONE	
WO 9822499 A	28-05-1998	CA 2190418 A AU 5044298 A	15-05-1998 10-06-1998